7

Modeling Tumor Angiogenesis in Zebrafish

Massimo M. Santoro
Molecular Biotechnology Center, University of Torino, Torino, Italy

1. Introduction

The process of angiogenesis is essential for tumor progression and metastasis. New pathways have been identified to play a critical role in promoting and regulating blood vessel formation both in embryogenesis and in pathophysiological conditions. These pathways provide potential molecular targets for anti-angiogenic therapies to treat cancer and other vascular diseases. The critical cellular targets of these therapies are vascular endothelial cells (ECs) and supporting mural cells or pericytes (MCs) that are recruited from surrounding healthy tissue to form new vessels in the growing tumor. A challenging task has always been to visualize these biological processes in vivo as well as to screen for drugs affecting these pathological pathways. In this context, the zebrafish model represents an emerging vertebrate system to study the tumor angiogenesis process and to better understand the modification of tumor microenvironment by anti-angiogenesis therapy. Can a small tropical fish help to better understand the tumor angiogenesis process and identify new therapies for tumor angiogenesis ? In this chapter we illustrate how the zebrafish has emerged as a novel in vivo cancer model to study tumor-induced neovascularization and metastases. In the transparent zebrafish embryos, invasion and migration of tumor cells, their circulation in the vascular system, as well as the formation tumor-induced neovascularization can all be followed with high resolution in real time. Importantly, these zebrafish models allow to quantitate both metastatic behavior of transplanted tumor cells and tumor-cell induced neovascularization. The zebrafish model has the advantage of being a vertebrate equipped with easy and powerful genetic and imaging tools to investigate the mechanisms of tumor development and progression. In particular the transparency of embryos and lately also adult are transforming this model system in the leading in vivo model for cancer biology and tumor angiogenesis.

2. The zebrafish model

The zebrafish (*Danio rerio*) system has emerged in the past years as an ideal vertebrate model organism in which to study a wide variety of biological processes (Thisse and Zon, 2002). Zebrafish is a small, freshwater teleost native of Ganges river in South-East India. Some of the advantages of the zebrafish animal model system, together with its small size and low cost, include fecundity, with each female capable of laying 200-300 eggs per week, external fertilization that permits manipulation of embryos ex utero, and rapid development of optically clear embryos, which allows the direct observation of developing internal

organs and tissues in vivo (Thisse and Zon, 2002). These attributes have led to the emergence of the zebrafish as a preeminent embryological model. The zebrafish has proven to be a powerful vertebrate model system for the genetic analysis of developmental pathways and has just started to be exploited as a model for human disease and clinical research (Lieschke and Currie, 2007; Skromne and Prince, 2008). If in the past 20 years zebrafish has served as an excellent model for understanding normal development using its powerful genetics and embryology, now is becoming a unique opportunity to uncover novel insights into the molecular genetics of human diseases.

2.1 Zebrafish and angiogenesis

The zebrafish system possesses many advantages for vascular studies. By 24 h after fertilization the zebrafish embryo has already developed a functional cardiovascular system (beating heart, aorta, cardinal vein and blood). Since the zebrafish embryo is relatively small and aquatic, zebrafish embryos are not completely dependent on a functional vascular system to continue to survive and develop. This happens because embryos receive enough oxygen by passive diffusion, thereby allowing a detailed analysis of animals with severe vascular defects (Stainier, 2001). Embryos without cardiac contraction or blood thus develop normally until their size outstrips diffusive oxygenation. This allows the consequences of genetic manipulation on cardiovascular development to be observed for longer than would be possible in mammals, in which abnormal heart or vascular development is fatal very early in development. By contrast avian and mammalian embryos die rapidly in the absence of a functional cardiovascular system and are not easy visualized internally without fixation and staining.

Until 5 days post fertilization, the embryos are nearly transparent, allowing in vivo visualization of any tissue without instrumentation or manipulation other than microscopy. This feature allows observation of the heart and blood vessels during development even at single cell resolution. The availability of the fertilized egg for injection with genetic constructs greatly facilitates the generation of tissue-specific transgenics (Kawakami, 2007). Such transgenesis usually uses a native tissue-specific promoter to drive expression of a fluorescent reporter protein, such as green fluorescent protein (GFP). Examples of cardiovascular transgenic lines include Fli1:GFP (expresses GFP in endothelial cells and some neural crest-derived cells) (Lawson and Weinstein, 2002), kdrl:GFP (expresses GFP localized to endothelial cell) (Jin et al., 2005), GATA1:dsRED (expresses dsRED in erythrocytes) (Traver et al., 2003), CD41:GFP (expresses GFP in thrombocytes) (Lin et al., 2005) and cmlc2:GFP (expresses GFP in cardiomyocytes) (Burns et al., 2005) and TGNL-Cherry (expresses cherry in smooth muscle cells)(Gays and Santoro, personal communication).

Coupled with the embryo's optical clarity, these transgenic lines allow observation of in vivo cellular behavior in a manner impossible in other models. The striking transparency of the embryos facilitates morphological observation of internal organs in vivo under a simple stereomicroscope, and at the single-cell level using confocal and single plan microscopy. Transparent zebrafish embryos are also well suited for in vivo time-lapse imaging. The fast acquisition speed of spinning disk and 2-photon confocal microscopy reduces the recording times significantly when millimeter-sized embryos need to be imaged at high resolution and at short time intervals. Light Sheet Fluorescence Microscopy could also be very useful in zebrafish (Keller et al., 2008). These attributes, that have led to the emergence of the zebrafish as a preeminent embryological model, including its capacity for gain- and loss-

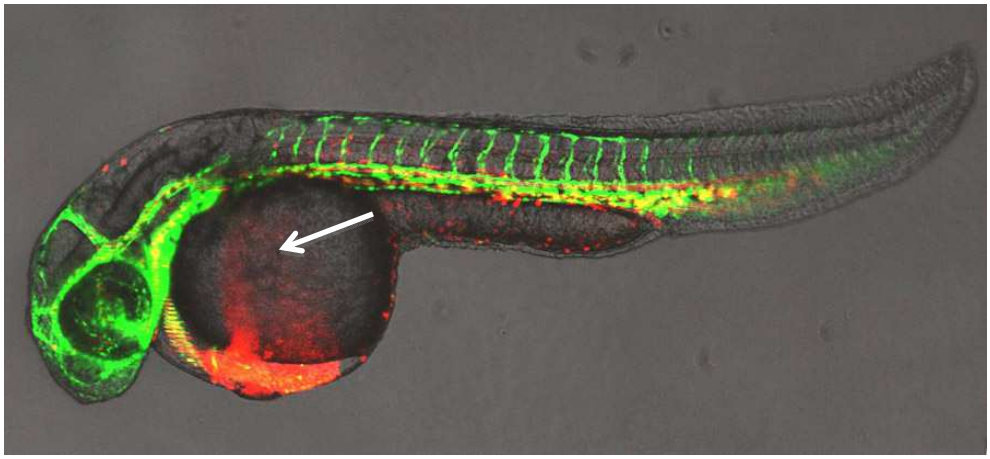


Fig. 1. Representative images of 30hpf transgenic Tg(kdrl:GFP^{s843},gata1:deRed^{sd2}) zebrafish embryos. Green fluorescent blood vessels and red fluorescent erythrocytes and macrophages are evident in all embryo. Arrow indicates point of tumor cells injections.

of-function studies, provides a unique opportunity to uncover novel insights into the molecular genetics of development and diseases. The zebrafish system also offers the opportunity to carry out forward genetic analysis to identify as yet unidentified loci/genes affecting vascular development (Patton and Zon, 2001). Different techniques exist to generate point mutations, deletions and mutagenic insertions, which can be bred to homozygosis in order to determine their phenotypic consequences. Zebrafish also allows the powerful combination of loss-of-function and gain-of-function analyses (Zon and Peterson, 2005). Altogether these data suggest the zebrafish system as an optimal model to study angiogenesis not only during development but also in pathological conditions such as tumor angiogenesis.

2.2 Zebrafish and cancer

The zebrafish (*Danio rerio*) has proven to be a powerful vertebrate model system for the genetic analysis of developmental pathways and is only beginning to be exploited as a model for human disease and clinical research. More recently, research with zebrafish has extended to model human diseases and to analyze the formation and functions of cell populations within organs (Dooley and Zon, 2000). This work has generated new human disease models and has begun to establish therapeutic possibilities, including genes that modify disease states and chemicals that rescue organs from disease. Zebrafish has recently entered the stage as a promising model system to study human cancer. This is largely due to the development of zebrafish transgenic lines expressing oncogenes and their amenability to genetic and pharmacological testing. Cancer progression in these animals recapitulates many aspects of human disease and opens the door for studies to identify genetic and chemical modifiers of cancer. The zebrafish is amenable to transgenic and genetic strategies that can be used to identify or generate zebrafish models of different types of cancer and may also present significant advantages for the discovery of tumor suppressor genes that promote tumorigenesis when inactivated by mutations (Amatruda et al., 2002). Importantly,

the transparency and accessibility of the zebrafish embryo and adult allows the unprecedented direct analysis of pathologic processes in vivo, in particular tumor angiogenesis (Moshal et al., 2010; Nicoli and Presta, 2007; Stoletov and Klemke, 2008). The attention has been further fueled by the development of xenograft models that allow the propagation and visualization of human cancer cells engrafted in optically transparent. The integration of zebrafish genetics with the large tool chest of reagents available to study human cancer cells provides a powerful new vertebrate model to visualize and dissect the mechanisms that drive cancer formation, angiogenesis and metastasis. Finally, zebrafish have many attributes that cancer researchers find attractive. Compared to mice, zebrafish require minimal care and are cost effective to maintain in the laboratory. A pair of adult fish produces several hundred fertilized eggs a week. The embryos develop externally and are transparent up to 1 month of age. Adult, casper mutant (noy$^{-/-}$; nacre$^{-/-}$) animals are also available that remain transparent throughout life and are amenable to in vivo transplantation studies (White et al., 2008). The remarkable transparency of zebrafish tissues allows direct imaging of cancer progression including cell invasion, intravasation, extravasation and angiogenesis (Rouhi et al., 2010b; Stoletov et al., 2007).

Ultimately, high-throughput modifier screens based on zebrafish cancer models can lead to the identification of chemicals or genes involved in the suppression or prevention of the malignant phenotype (Lieschke and Currie, 2007). The identification of small molecules or gene products through such screens will serve as ideal entry points for novel drug development for cancer treatment. Here we focus on the current technology that takes advantage of the zebrafish model system to advance our understanding of the genetic basis of tumor angiogenesis and its treatment.

2.3 Molecular mechanisms involved in angiogenesis

The vascular system is the first organ to form and function during embryogenesis. Vascular development begins with the organization of endothelial cells (ECs) into a primitive vascular plexus that becomes progressively remodeled to ultimately form a complex vascular network, a process called angiogenesis (Carmeliet, 2003; Cleaver and Melton, 2003; Red-Horse et al., 2007). Afterwards, the developing vessels differentiate and mature by generating extracellular matrices, by expressing specific cell-cell molecules, and by recruiting mural cells (MCs)(pericytes and vascular smooth muscle cells) in a process called vascular maturation. MCs are recruited to the endothelial vasculature and sheathe it, providing support and contractility to the mature vascular system (Bergers and Song, 2005; Carmeliet, 2005). Formation and stabilization of the vascular system is essential for proper development of vertebrate embryos, as well as for the survival of adults. Mature vessels continue to provide metabolic homeostasis by supplying oxygen and nutrients and removing metabolic wastes. Several molecular mechanisms are involved during the vascular maturation process, including signaling pathways (e.g. PDGF, Angiopoietins, TGFb1, DlGF, S1P-EDG1), cell-cell and cell-matrix interactions (e.g. cadherins, connexins, integrins, MMPs, PAI1). Formation of new blood vessels is desirable for regenerative purposes, such as during tissue healing or transplantation, but can be pathological, as in diabetic retinopathy and cancer.

Several studies in zebrafish have identified new mechanism involved in angiogenesis. It has been demonstrated that numerous angiogenic factors not only induce angiogenesis but also function as EC survival factors. Using genetic studies in zebrafish, we previously discovered that Birc2/cIap1, an inhibitor of apoptosis (IAP), is critical for the survival of endothelial

cells, which line the inner portion of blood vessels (Santoro et al., 2007). Apart from the relevance of these findings for improving understanding of vascular development, they may create new opportunities for further development of anti- angiogenic drug therapies. In fact, several reports demonstrate that IAP antagonist potentiate apoptosis in cancer cells by promoting induction of auto-ubiquitination and degradation of cIAPs, which culminates in TNFα-mediated cell death suggesting an important role of this pathway in tumor angiogenesis (Wu et al., 2005).
Non-coding RNAs called microRNAs also modulate the response of the vascular endothelium to angiogenic stimuli. Recently different microRNA (e.g. miRNA126, and miRNA296 have been found to be associated with angiogenesis in zebrafish (Fish and Srivastava, 2009). However, many other mechanisms remain to be discovered.

2.4 Tumor angiogenesis

A tumor consists of a population of rapidly dividing and growing cancer cells. Cancer cells have lost their ability to divide in a controlled fashion as a consequence tumor cells rapidly accumulate mutations that allow cancer cells (or sub-populations of cancer cells within a tumor) to grow more (Carmeliet, 2003; Folkman, 2007). Tumors cannot grow beyond a certain size, generally 1–2 mm^3 due to a lack of oxygen and other essential nutrients. Tumors cells have then have acquired a specific feature that is to induce blood vessel growth (a process called tumor angiogenesis) by secreting various endothelial growth factors. Endothelial specific growth factors such as bFGF and VEGF-A can induce capillary growth into the tumor that in turn allow tumor expansion. Tumor angiogenesis is a necessary and required step for transition from a small harmless cluster of cells to a large tumor. Angiogenesis is also required for the spread of a tumor, or metastasis. Single cancer cells can break away from an established solid tumor, enter the blood vessel, and be carried to a distant site, where they can implant and begin the growth of a secondary tumor. Evidence now suggests the blood vessel in a given solid tumor may, in fact, be mosaic vessels, composed of endothelial cells, mural cells and neoplastic cells. The subsequent growth of such metastases will also require a supply of nutrients and oxygen and a waste disposal pathway.
Endothelial cells have long been considered genetically more stable than cancer cells. This genomic stability confers an advantage to targeting endothelial cells using antiangiogenic therapy, compared to chemotherapy directed at cancer cells, which rapidly mutate and acquire 'drug resistance' to treatment. For this reason, endothelial cells are thought to be an ideal target for therapies directed against them (Folkman, 2007). Tumor blood vessels have perivascular detachment, vessel dilation, and irregular shape. It is believed that tumor blood vessels are not smooth like normal tissues, and are not ordered sufficiently to give oxygen to all of the tissues. Endothelial precursor cells are organized from bone marrow, which are then integrated into the growing blood vessels. Afterwards endothelial cells differentiate and migrate into perivascular space, providing nutrients that allow neoplastic cells to growth the tumor mass. In this process VEGF-A plays a crucial role since it is important for *denovo* formation of new blood vessels at sites of tumor formation allowing cancer cell to growth (Bergers and Hanahan, 2008).
Tumor angiogenesis is one of the most prominent mechanisms driving tumor development and progression. Tumor angiogenesis research is a cutting-edge field in cancer research and recent evidence also suggests traditional therapies, such as radiation therapy, may actually work in part by targeting the genomic stable endothelial cell compartment, rather than the

genomic unstable tumor cell compartment. New blood vessel formation is a relatively fragile process, subject to disruptive interference at several levels. In short, the therapy is the selection agent that is being used to kill a cell compartment. Tumor cells evolve resistance rapidly due to rapid generation time (days) and genomic instability (variation), whereas endothelial cells are a good target because of a long generation time (months) and genomic stability (low variation). As a consequence crucial targets for therapeutic intervention have been identified and validated. Based on these efforts and achievements, targeted drug development programs have been implemented to interfere with tumor angiogenesis as an attractive strategy in cancer treatment. As a promising result the first targeted anti-angiogenic drugs have been approved for a variety of solid metastasizing cancers. The first generation of these molecules targets the two most prominent regulatory components of tumor angiogenesis: the vascular endothelial growth factor (VEGF-A), produced by tumor cells, and the VEGF-A receptor tyrosine kinase, which is expressed on vascular endothelial cells. Beyond the VEGF receptor system, additional tumor-angiogenic systems are presented as new potential targets for anti-angiogenic therapy (Bergers and Benjamin, 2003).

Over the last decades studies of tumor angiogenesis have concentrated mainly on the endothelial cells component, while the interest for mural cells has lagged behind (Bergers and Hanahan, 2008). Mural cells (MCs)(aka pericytes or vascular smooth muscle cells) are perivascular cells that wrap around blood capillaries. They communicate with endothelial cells by direct physical contact through a jointly synthesized basement membrane and reciprocal paracrine signalling. MCs and endothelial cells are thereby interdependent and, as such, defects in either endothelial cells or pericytes can affect the vascular system. Mural cells have various demonstrable functions in different physiological contexts, including stabilization and homeostatic regulation of mature blood vessels; facilitation of vessel maturation in the context of neovascularization; provision by their intimate association of endothelial cell survival signals; and limitation of cell transit across the vascular wall. The functional significance of mural cells in development is underscored by genetic depletion or disruption of mural cells association with the developing vasculature, which results in blood vessel dilation, widespread microvascular leakage and subsequent lethality during late gestation (Bergers and Song, 2005; Conway et al., 2001; Red-Horse et al., 2007). In tumors, MCs are typically less abundant and more loosely attached to blood vessels than in normal tissues, but their association is still important, as shown in a growing body of experimental evidence which indicates that pericytes help to maintain the integrity and functionality of the tumor vasculature (Armulik et al., 2005; Majesky, 2007). Interestingly, a growing body of evidence indicates that MCs, the periendothelial support cells of the microvasculature, are also important cell constituents of the aberrant tumor vasculature. When therapies impair neovascularization and/or elicit vascular regression, some tumors evidently rely on pericytes to help keep a core of pre-existing blood vessels alive and functional. This concept has evolved from the observation by several groups that, although inhibition of VEGF signalling can lead to substantial reduction in tumor vascularization, distinctive functional vessels remain that are slim and tightly covered with pericytes. These observations suggest that endothelial cells can induce MCs recruitment to protect themselves from death consequent to the lack of the crucial tumor-derived survival signals conveyed by VEGF-A. This hypothesis is supported by the findings that tumor vessels lacking adequate MCs coverage are more vulnerable to VEGF-A inhibition and that tumor MCs, which are juxtacrine to endothelial cells, express appreciable levels of VEGF-A and potentially other factors that support endothelial cell survival. The understanding of these mechanisms

represents an important first step to elucidate pathways leading to many vascular-associated disorders, including tumor angiogenesis. The integration of zebrafish transgenic technology with human cancer biology may aid in the development of cancer models that target specific organs, tissues, or cell types within the tumors. Zebrafish could also provide a cost-effective means for the rapid development of therapeutic agents directed at blocking human cancer progression and tumor-induced angiogenesis (Amatruda et al., 2002).

2.5 Zebrafish as a model to study tumor angiogenesis

It has already been shown that blood vessels can grow by positional cues as well as secreted factors from other tissues. During pathological states, solid tumors use molecular mechanisms that are involved during normal angiogenesis to promote the disease state and establish a microenvironment to render normally quiescent endothelial cells to proliferate. In this respect, tumor tissue secretes angiogenic factors that can regulate blood vessel development (Bergers and Benjamin, 2003). However, not much is known about the cellular signals that initiate or establish cross talk between the tumor and the stromal cells. The study of the cellular interaction of tumor cells with its surrounding endothelial cells is expected to aid in the discovery of genes/pathways involved in the process of blood vessel formation in vivo. The microenvironment of a host-tumor interface has a profound influence on disease progression and has the potential for cancer therapy. Despite the importance of tumor-stromal interactions, there is a limited understanding of signaling cross talk between the tumor and the host microenvironment. Much of the information regarding the signaling networks comes from cell culture studies; however, the main drawback of this approach is the difficulty in extrapolating these findings to the whole organism. Thus, there is a need to develop suitable whole animal models to study the host- tumor interface.

In this respect, the zebrafish xenotransplantation system represents a novel model for defining tumor angiogenesis by the means of high-throughput manipulation of host environment, via morpholino knockdown of genetic pathways and treatment with small molecule inhibitors (Haldi et al., 2006; Nicoli et al., 2007; Rouhi et al., 2010a; Stoletov et al., 2007). Although embryonic and tumor vasculatures have morphological differences, they are mechanistically similar in the process of angiogenesis; therefore, studying the zebrafish xeno-transplantation model will allow us to better understand the impact of manipulation of the host microenvironment on tumor angiogenesis.

2.6 The xenotranplant experiments

The xenotransplantation studies conducted in mice and humans are limited by difficulties for direct observation in real time of cellular and signaling events in the context of the whole organism. Therefore, the future challenge will be to analyze the functional role of signaling interaction in modulating tumor angiogenesis by the host environment and determining its relationship with the inflammatory response and tumor cell metastasis using zebrafish as a transparent and tractable whole animal model system. In this regard, the use of transparent adult zebrafish casper mutant with fluorescently tagged blood vessels and myeloid-specific transgenic zebrafish will be advantageous to study cellular responses in real time (White et al., 2008). The other advantages include the speed of analysis for tumor angiogenesis (24-48 hours) and the ability to perform whole- mount in situ gene expression analyses in heterologous tumor cells vs the tumor-induced gene expression in the surrounding host environment leading to the attractiveness and appropriateness of the zebrafish model to study tumor angiogenesis. Various groups have shown that human cancer cells when

grafted in early zebrafish embryos (1/2-day-old) may promote neoangiogenesis from embryos vessels and spread of metastasis in all embryos, thereby validating the xenotransplantation procedure to study tumor angiogenic and metastatic processes in zebrafish (Haldi et al., 2006; Nicoli et al., 2007; Stoletov et al., 2007) (Topczewska et al., 2006; Marques et al., 2009). The major advantage of the xenotransplantation in early embryos is the immature immune system that permits cancer cell engraftment without rejection. The studies by Haldi and collaborators demonstrated the feasibility of using 2 days postfertilization (dpf) zebrafish for xeno-transplantation where the fluorescently labeled human carcinoma was grafted into the yolk sac and observed until 7 days postinjection for cancer cell proliferation, migration, infiltration of tumor masses by endothelial cells, vessel remodeling, and stimulation of angiogenesis (Haldi et al., 2006). This technique represents a promising vertebrate model to study tumor-host microenvironment and to screen for antiangiogenic compounds. Later on, Presta and collaborators have refined the xenotransplantation process by transplanting tumor cells directly into the perivitelline space between the periderm and the yolk syncytial layer of the zebrafish embryos at 48 hours postfertilization and observed the neoangiogenic response originating from the subintestinal vessel plexus (Nicoli et al., 2007). Using the early zebrafish xenotransplantation model, Cao and collaborators monitored the dissemination of single-tumor cells from the primary sites and recapitulated early stages of clinical metastasis (Rouhi et al., 2010b). They concluded that hypoxia and VEGF-A-induced neovascularization promoted tumor invasion and metastasis by increasing dissemination of tumor cells into the circulation. Using the zebrafish xenotransplantation, Vlecken and Bagowski documented the significance of LIM domain kinases 1 and 2 signal molecules in tumor metastasis and angiogenesis of human pancreatic cancer cell lines and suggested that simultaneous targeting of both LIM domain kinases could inhibit tumor progression and metastasis (Vlecken and Bagowski, 2009). Hence, zebrafish early xenotransplantation may represent a powerful tool to understand how the angiogenic phenotype of the cancer culminates in metastatic spread of tumor and will be beneficial to study the molecular mechanism of antiangiogenesis therapy.

Later on, Stoletov and colleques successfully transplanted human cancer cells in a 30-day-old transparent zebrafish and studied the dynamics of microtumor formation and angiogenesis, thereby making this an excellent model appropriate to study the basics of vessel remodeling and alteration in subcellular compartments during tumorigenesis (Stoletov et al., 2007). It is not fully understood whether the new vessels triggered by human cancer cells are the result of redirection of the preexisting vessels and/or recruiting from the circulating endothelial cells. Therefore, using this late zebrafish xenotransplantation model will be better suited to investigate the origin of these endothelial cells in the transparent zebrafish host in which the vasculature has already established. The other advantages of using late xenotransplantation include easy interpretation of tumor-induced vascular effects, since the major organs in the juvenile fish including the vasculature have fully formed including smooth muscle cells and pericytes that can play a key role in tumor angiogenesis and it eliminates the concern related to the developmental defects due to embryologic manipulation. On the other hand, a limitation for this late model is the requirement of chemical suppression of the host immune function for successful grafting of the cancer cells. Recently, the generation of homozygous diploid clonal zebrafish lines by heat shock method made it possible to perform transplantation of hepatic tumor from one fish to another without rejection of the graft and without compromised by immunosuppression or sublethal γ-irradiation (Mizgireuv and Revskoy, 2006). However,

clonal zebrafish model is suitable for studying various cancer types of zebrafish origin; it cannot be used as a host for transplanting human cancer cells without immunosuppression. To date, the early events that trigger vascular remodeling and tumor angiogenesis during tumor formation are not known. In addition, micrometastasis is a major cause of deaths in cancer patients. In this regard, a 30-day-old zebrafish xenotransplantation might also represent an adequate model to dissect the molecular and cellular mechanisms leading to micro-tumor formation.

2.7 Tumor angiogenesis-associated molecular mechanisms in zebrafish

Novel function of important genes associated with the process of tumor angiogenesis has been identified recently using the zebrafish model, including galectin-1 (Thijssen et al., 2006), Chemokine receptor 7 (Miao et al., 2007), LIM domain kinase 1 and 2 (Vlecken and Bagowski, 2009), angiomodulin (Hooper et al., 2009), Hypoxia inducible factor (Lee et al., 2009). In the past we identified a new molecule, Birc2/cIAP, important for endothelial survival in normal and pathological conditions (Gyrd-Hansen et al., 2008; Santoro et al., 2007). This study has implications for future design of antiangiogenic therapy. Available antiangiogenic agents (all VEGF inhibitors) induce endothelial cell apoptosis in existing vessels. Hence, additional antiangiogenic agents with complementary mechanisms are required. Because Birc2 has enzymatic activity, it might become an attractive target for drug development. We are currently testing a specific cIAP antogonists (BV6 and derivates) in endothelial cells in zebrafish (Varfolomeev et al., 2007). These small molecules potentiate apoptosis in cancer cells by promoting caspases activation. We will test these compounds and its derivates on out xenograft model of tumor angiogenesis in zebrafish. During the last years we also worked on characterization of mural cells (pericytes and vascular smooth muscle cells) (Santoro et al., 2009). Identify the molecular mechanisms involved in mural cell differentiation is very important to target tumor angiogenesis because it is known that tumor endothelial cells required pericytes/mural cells to survive (von Tell et al., 2006). Better understanding of the molecular genetic of mural cells may lead to the identification of new important targets for tumor angiogenesis and help to design anti-angiogenic drugs.

Nicoli et al., showed that morpholino knockdown of VE-cadherin expression selectively prevented tumor cell-induced angiogenesis by FGF2, but not normal vessel development including formation of the intersegmental and subintestinal vessels (Nicoli et al., 2007). These findings are surprising in light of the fact that VE-cadherin null mice display several vascular defects in vessel assembly that cause embryonic lethality at day 9.5 (Carmeliet et al., 1999). The apparent discrepancy in these studies may be due to differences in the vascular programs utilized by fish and mammals. It is also possible that tumor-induced vessel formation in zebrafish may be uniquely sensitive to perturbation of VE-cadherin expression or that the same programs that drive tumor-induced angiogenesis are different from those that drive developmental vasculogenesis.

Recently, Stoletov and Klemke demonstrate that highly invasive human cancer cell lines such as HT1080 or Hep3 are also invasive in zebrafish host while low invasive human cells as MDA-435 do not disseminate in the host zebrafish tissue (Stoletov and Klemke, 2008). Human cancer cells overexpressing RhoC or src display increased cell dissemination when transplanted in zebrafish demonstrating that the molecules (ECM, growth factors, MMPs) and mechanisms playing a role in cancer cell-host tissue interaction are highly conserved between human and zebrafish.

3. Conclusions

Zebrafish recently entered the stage as a promising model system to study human cancer. This has been largely due to the development of transgenic and xenograft models of cancer, and their amenability to genetic and pharmacological testing. Cancer progression in these animals recapitulates many aspects of human disease and opens the door for studies to identify genetic and chemical modifiers of cancer. There are general advantages and limitations of using zebrafish as an in vivo model to study tumor angiogenesis. These include:

Advantages

- Zebrafish husbandry: inexpensive to obtain and maintain large number of adult and embryo zebrafish.
- Generation of larvae with deletion or overexpression of specific genes can be easily accomplished using available tools (e.g. morpholinos, RNA, mimics).
- Genetically manipulated zebrafish strains that are defective or have acquired functions of certain gene products are available (e.g. zinc-fingers, ENU, tilling).
- Transgenic zebrafish lines that express reporter genes in particular cell types are also available in the scientific community.
- Addition of active chemical stimulators or inhibitors to the water enables analysis of intervention of these compounds on physiological and pathological processes.
- Turnover time for experiments is relatively short.
- Optical clarity of zebrafish embryos allows visualization of vascular and hematopoietic cells as well as tumor cell dissemination in living animals.
- Zebrafish embryos allows implantation of mammalian tumor cells, including human and mouse tumor cells, due to the absence of a functional immune system at this stage

Limitations

- Few antibodies against zebrafish proteins are available so far. For this reason it is difficult to perform immunofluorescence analyses in zebrafish samples.
- Due relative small size of zebrafish larvae, you need skillful and careful trainees to perform experiments.
- As most mammalian tumors grow at 37 °C, it is difficult to study the process of xenograft tumor growth at the optimal temperature.
- Microinjection of tumor cells into the perivitelline space of a large number of zebrafish embryos is a tedious procedure and requires highly skillful micro-operations.

These models are already being utilized by academia and industry to search for genetic and chemical modifiers of cancer with success. The attention has been further stimulated by the amenability of zebrafish to pharmacological testing and the superior imaging properties of fish tissues that allow visualization of cancer progression and angiogenesis in live animals.

Here we have described how the zebrafish/tumor xenograft model is becoming an emerging vertebrate system to study tumor angiogenesis. In particular this model become very interesting to analyze the molecular and cellular mechanisms of tumor angiogenesis in real time. Within the past several years, zebrafish has shown great promise to become a powerful animal model to study cancer progression and several laboratories have focused on zebrafish and tumor biology. However, despite a significant progress, more work is required to fully explore how closely these processes in zebrafish may parallel mammalian

cancer mechanisms and how well they might be translated to human disease. This improvement will require more specific comparative studies among transgenic and xenograft zebrafish and mouse/mammalian models of cancer/tumor angiogenesis and their relevance to the disease. When all these studies and analyses will be performed the zebrafish model will have the full potential to be used and embraced by the cancer research community.

4. Acknowledgment

I apologize to the many researchers whose work was not cited in this review due to space limitations. We would like to thank all members of Santoro laboratory for support and discussion as well as Ellen Jane Corcoran. Work in Santoro 's laboratory is supported by grants from HFSP, Marie Curie IRG, Telethon and AIRC.

5. References

Amatruda, J. F., Shepard, J. L., Stern, H. M. and Zon, L. I. (2002). Zebrafish as a cancer model system. *Cancer Cell* 1, 229-31.

Armulik, A., Abramsson, A. and Betsholtz, C. (2005). Endothelial/pericyte interactions. *Circ Res* 97, 512-23.

Bergers, G. and Benjamin, L. E. (2003). Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 3, 401-10.

Bergers, G. and Hanahan, D. (2008). Modes of resistance to anti-angiogenic therapy. *Nat Rev Cancer* 8, 592-603.

Bergers, G. and Song, S. (2005). The role of pericytes in blood-vessel formation and maintenance. *Neuro Oncol* 7, 452-64.

Burns, C. G., Milan, D. J., Grande, E. J., Rottbauer, W., MacRae, C. A. and Fishman, M. C. (2005). High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat Chem Biol* 1, 263-4.

Carmeliet, P. (2003). Angiogenesis in health and disease. *Nat Med* 9, 653-60.

Carmeliet, P. (2005). Angiogenesis in life, disease and medicine. *Nature* 438, 932-6.

Cleaver, O. and Melton, D. A. (2003). Endothelial signaling during development. *Nat Med* 9, 661-8.

Conway, E. M., Collen, D. and Carmeliet, P. (2001). Molecular mechanisms of blood vessel growth. *Cardiovasc Res* 49, 507-21.

Coultas, L., Chawengsaksophak, K. and Rossant, J. (2005). Endothelial cells and VEGF in vascular development. *Nature* 438, 937-45.

Dooley, K. and Zon, L. I. (2000). Zebrafish: a model system for the study of human disease. *Curr Opin Genet Dev* 10, 252-6.

Duval, H., Harris, M., Li, J., Johnson, N. and Print, C. (2003). New insights into the function and regulation of endothelial cell apoptosis. *Angiogenesis* 6, 171-83.

Duval, H., Johnson, N., Li, J., Evans, A., Chen, S., Licence, D., Skepper, J., Charnock-Jones, D. S., Smith, S. and Print, C. (2007). Vascular development is disrupted by endothelial cell-specific expression of the anti-apoptotic protein Bcl-2. *Angiogenesis* 10, 55-68.

Eimon, P. M., Kratz, E., Varfolomeev, E., Hymowitz, S. G., Stern, H., Zha, J. and Ashkenazi, A. (2006). Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. *Cell Death Differ* 13, 1619-30.

Fish, J. E. and Srivastava, D. (2009). MicroRNAs: opening a new vein in angiogenesis research. *Sci Signal* 2, pe1.

Folkman, J. (2007). Angiogenesis: an organizing principle for drug discovery? *Nat Rev Drug Discov* 6, 273-86.

Gyrd-Hansen, M., Darding, M., Miasari, M., Santoro, M. M., Zender, L., Xue, W., Tenev, T., da Fonseca, P. C., Zvelebil, M., Bujnicki, J. M. et al. (2008). IAPs contain an evolutionarily conserved ubiquitin-binding domain that regulates NF-kappaB as well as cell survival and oncogenesis. *Nat Cell Biol* 10, 1309-17.

Haldi, M., Ton, C., Seng, W. L. and McGrath, P. (2006). Human melanoma cells transplanted into zebrafish proliferate, migrate, produce melanin, form masses and stimulate angiogenesis in zebrafish. *Angiogenesis* 9, 139-51.

Hooper, A. T., Shmelkov, S. V., Gupta, S., Milde, T., Bambino, K., Gillen, K., Goetz, M., Chavala, S., Baljevic, M., Murphy, A. J. et al. (2009). Angiomodulin is a specific marker of vasculature and regulates vascular endothelial growth factor-A-dependent neoangiogenesis. *Circ Res* 105, 201-8.

Jin, S. W., Beis, D., Mitchell, T., Chen, J. N. and Stainier, D. Y. (2005). Cellular and molecular analyses of vascular tube and lumen formation in zebrafish. *Development* 132, 5199-209.

Kawakami, K. (2007). Tol2: a versatile gene transfer vector in vertebrates. *Genome Biol* 8 Suppl 1, S7.

Keller, P. J., Schmidt, A. D., Wittbrodt, J. and Stelzer, E. H. (2008). Reconstruction of zebrafish early embryonic development by scanned light sheet microscopy. *Science* 322, 1065-9.

Lawson, N. D. and Weinstein, B. M. (2002). In vivo imaging of embryonic vascular development using transgenic zebrafish. *Dev Biol* 248, 307-18.

Lee, B. W., Chae, H. Y., Tuyen, T. T., Kang, D., Kim, H. A., Lee, M. and Ihm, S. H. (2009). A comparison of non-viral vectors for gene delivery to pancreatic beta-cells: delivering a hypoxia-inducible vascular endothelial growth factor gene to rat islets. *Int J Mol Med* 23, 757-62.

Lieschke, G. J. and Currie, P. D. (2007). Animal models of human disease: zebrafish swim into view. *Nat Rev Genet* 8, 353-67.

Lin, H. F., Traver, D., Zhu, H., Dooley, K., Paw, B. H., Zon, L. I. and Handin, R. I. (2005). Analysis of thrombocyte development in CD41-GFP transgenic zebrafish. *Blood* 106, 3803-10.

Majesky, M. W. (2007). Developmental basis of vascular smooth muscle diversity. *Arterioscler Thromb Vasc Biol* 27, 1248-58.

Marques, I. J., Weiss, F. U., Vlecken, D. H., Nitsche, C., Bakkers, J., Lagendijk, A. K., Partecke, L. I., Heidecke, C. D., Lerch, M. M. and Bagowski, C. P. (2009). Metastatic behaviour of primary human tumours in a zebrafish xenotransplantation model. *BMC Cancer* 9, 128.

Mazzone, M., Ruiz de Almodovar, C. and Carmeliet, P. (2007). Building in resistance to endothelial cell death. *Nat Genet* 39, 1308-9.

Miao, Z., Luker, K. E., Summers, B. C., Berahovich, R., Bhojani, M. S., Rehemtulla, A., Kleer, C. G., Essner, J. J., Nasevicius, A., Luker, G. D. et al. (2007). CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci U S A* 104, 15735-40.

Micheau, O. and Tschopp, J. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell* 114, 181-90.

Mizgireuv, I. V. and Revskoy, S. Y. (2006). Transplantable tumor lines generated in clonal zebrafish. *Cancer Res* 66, 3120-5.

Moshal, K. S., Ferri-Lagneau, K. F. and Leung, T. (2010). Zebrafish model: worth considering in defining tumor angiogenesis. *Trends Cardiovasc Med* 20, 114-9.

Nicoli, S. and Presta, M. (2007). The zebrafish/tumor xenograft angiogenesis assay. *Nat Protoc* 2, 2918-23.

Nicoli, S., Ribatti, D., Cotelli, F. and Presta, M. (2007). Mammalian tumor xenografts induce neovascularization in zebrafish embryos. *Cancer Res* 67, 2927-31.

Patton, E. E. and Zon, L. I. (2001). The art and design of genetic screens: zebrafish. *Nat Rev Genet* 2, 956-66.

Red-Horse, K., Crawford, Y., Shojaei, F. and Ferrara, N. (2007). Endothelium-microenvironment interactions in the developing embryo and in the adult. *Dev Cell* 12, 181-94.

Rouhi, P., Jensen, L. D., Cao, Z., Hosaka, K., Lanne, T., Wahlberg, E., Steffensen, J. F. and Cao, Y. (2010a). Hypoxia-induced metastasis model in embryonic zebrafish. *Nat Protoc* 5, 1911-8.

Rouhi, P., Lee, S. L., Cao, Z., Hedlund, E. M., Jensen, L. D. and Cao, Y. (2010b). Pathological angiogenesis facilitates tumor cell dissemination and metastasis. *Cell Cycle* 9, 913-7.

Sakamaki, K. (2004). Regulation of endothelial cell death and its role in angiogenesis and vascular regression. *Curr Neurovasc Res* 1, 305-15.

Santoro, M. M., Pesce, G. and Stainier, D. Y. (2009). Characterization of vascular mural cells during zebrafish development. *Mech Dev* 126, 638-49.

Santoro, M. M., Samuel, T., Mitchell, T., Reed, J. C. and Stainier, D. Y. (2007). Birc2 (cIap1) regulates endothelial cell integrity and blood vessel homeostasis. *Nat Genet*.

Skromne, I. and Prince, V. E. (2008). Current perspectives in zebrafish reverse genetics: moving forward. *Dev Dyn* 237, 861-82.

Stainier, D. Y. (2001). Zebrafish genetics and vertebrate heart formation. *Nat Rev Genet* 2, 39-48.

Stoletov, K. and Klemke, R. (2008). Catch of the day: zebrafish as a human cancer model. *Oncogene* 27, 4509-20.

Stoletov, K., Montel, V., Lester, R. D., Gonias, S. L. and Klemke, R. (2007). High-resolution imaging of the dynamic tumor cell vascular interface in transparent zebrafish. *Proc Natl Acad Sci U S A* 104, 17406-11.

Thijssen, V. L., Postel, R., Brandwijk, R. J., Dings, R. P., Nesmelova, I., Satijn, S., Verhofstad, N., Nakabeppu, Y., Baum, L. G., Bakkers, J. et al. (2006). Galectin-1 is essential in tumor angiogenesis and is a target for antiangiogenesis therapy. *Proc Natl Acad Sci U S A* 103, 15975-80.

Thisse, C. and Zon, L. I. (2002). Organogenesis--heart and blood formation from the zebrafish point of view. *Science* 295, 457-62.

Topczewska, J. M., Postovit, L. M., Margaryan, N. V., Sam, A., Hess, A. R., Wheaton, W. W., Nickoloff, B. J., Topczewski, J. and Hendrix, M. J. (2006). Embryonic and tumorigenic pathways converge via Nodal signaling: role in melanoma aggressiveness. *Nat Med* 12, 925-32.

Traver, D., Paw, B. H., Poss, K. D., Penberthy, W. T., Lin, S. and Zon, L. I. (2003). Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nat Immunol* 4, 1238-46.

Varfolomeev, E., Blankenship, J. W., Wayson, S. M., Fedorova, A. V., Kayagaki, N., Garg, P., Zobel, K., Dynek, J. N., Elliott, L. O., Wallweber, H. J. et al. (2007). IAP antagonists induce autoubiquitination of c-IAPs, NF-kappaB activation, and TNFalpha-dependent apoptosis. *Cell* 131, 669-81.

Varfolomeev, E. E., Schuchmann, M., Luria, V., Chiannilkulchai, N., Beckmann, J. S., Mett, I. L., Rebrikov, D., Brodianski, V. M., Kemper, O. C., Kollet, O. et al. (1998). Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity* 9, 267-76.

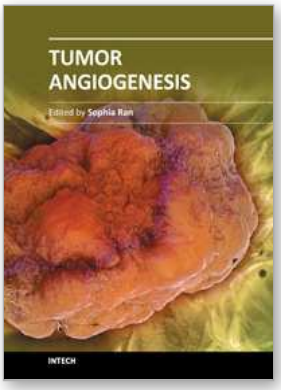
Vlecken, D. H. and Bagowski, C. P. (2009). LIMK1 and LIMK2 are important for metastatic behavior and tumor cell-induced angiogenesis of pancreatic cancer cells. *Zebrafish* 6, 433-9.

von Tell, D., Armulik, A. and Betsholtz, C. (2006). Pericytes and vascular stability. *Exp Cell Res* 312, 623-9.

White, R. M., Sessa, A., Burke, C., Bowman, T., LeBlanc, J., Ceol, C., Bourque, C., Dovey, M., Goessling, W., Burns, C. E. et al. (2008). Transparent adult zebrafish as a tool for in vivo transplantation analysis. *Cell Stem Cell* 2, 183-9.

Wu, C. J., Conze, D. B., Li, X., Ying, S. X., Hanover, J. A. and Ashwell, J. D. (2005). TNF-alpha induced c-IAP1/TRAF2 complex translocation to a Ubc6-containing compartment and TRAF2 ubiquitination. *Embo J* 24, 1886-98.

Zon, L. I. and Peterson, R. T. (2005). In vivo drug discovery in the zebrafish. *Nat Rev Drug Discov* 4, 35-44.



Tumor Angiogenesis
Edited by Dr. Sophia Ran

ISBN 978-953-51-0009-6
Hard cover, 296 pages
Publisher InTech
Published online 17, February, 2012
Published in print edition February, 2012

Tumor angiogenesis is the main process responsible for the formation of new blood vessels that promote tumor growth and metastasis. This process is driven by potent pro-angiogenic factors that are predominant in the tumor environment and are produced by both malignant cells and the host cells recruited to the tumor site. Tumor environment is characterized by the imbalance between pro-angiogenic and anti-angiogenic factors, which drives the construction of numerous but structurally defective vessels. These poorly perfused and abnormal vessels significantly contribute to the tumor pathology not only by supporting the expansion of the tumor mass but also by promoting chronic inflammation, enhancing thrombosis, impeding drug delivery, and disseminating tumor cells. These problems associated with tumor vasculature continue to attract great attention of scientists and clinicians interested in advancing the understanding of tumor biology and development of new drugs. This book complies a series of reviews that cover a broad spectrum of current topics related to the pathology of tumor blood vessels including mechanisms inducing new vessels, identification of new targets for inhibition of tumor angiogenesis, and potential clinical use of known and novel anti-angiogenic therapies. The book provides an update on tumor angiogenesis that could be useful for oncologists, cancer researchers and biologists with interests in vascular and endothelial cell behavior in the context of cancer.

How to reference
In order to correctly reference this scholarly work, feel free to copy and paste the following:

Massimo M. Santoro (2012). Modeling Tumor Angiogenesis in Zebrafish, Tumor Angiogenesis, Dr. Sophia Ran (Ed.), ISBN: 978-953-51-0009-6, InTech, Available from: http://www.intechopen.com/books/tumor-angiogenesis/modeling-tumor-angiogenesis-in-zebrafish

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821